Histamine H_3 receptors expressed in ventral horns modulate spinal motor output

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Abstract (135 words)

Motoneuron activity is modulated by histamine receptors. While H_1 and H_2 receptors have been widely explored, H_3 histamine receptors (H_3Rs) have not been sufficiently characterized. This paper targets the effects of the selective activation of H_3Rs and their expression on the membranes of large ventral horn cells. The application of selective pharmacological agents to spinal cords isolated from neonatal rats was used to identify the presence of functional H_3Rs on the membrane of physiologically identified lumbar motoneurons. Intra and extracellular recordings revealed that H_3R agonist, α-methylhistamine, depolarized both single motoneurons and VRs, even in the presence of tetrodoxin (TTX), an effect prevented by H_3R antagonist, thioperamide. Finally, immunohistochemistry located the expression of H_3Rs on a subpopulation of large cells in lamina IX. This study identifies H_3Rs as a new exploitable pharmacological target against motor disturbances.

Keywords: motoneurons, motor pools, motor reflexes, spontaneous activity

Abbreviations: CV, cresyl violet; DRG, dorsal root ganglion; H_3Rs, H_3 histamine receptors; P, postnatal; VR, ventral root

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(2005 words)

Histamine in the spinal cord is released by fibers descending from the tuberomamillary nucleus of the posterior hypothalamus, the exclusive location for histaminergic neurons (Haas et al., 2008). Histamine-immunoreactive spinal fibers are located around the central canal (Inagaki et al., 1988) and scattered in the anterior horn of the lumbar cord, mainly condensed in lamina X (Seybold, 1985).

In the mammalian spinal cord, the rhythmic patterns generated by neural circuits are modulated by histamine (Coslovich et al., 2018). Four metabotropic histamine receptors have been reported in the CNS (H_{1-4}; Haas et al., 2008) and are also identified in the spinal cord (Taylor et al., 1982 for H_1 subtype; Murakami et al., 1999 for H_2; Cannon et al., 2007 for H_3; Strakhova et al., 2009 for H_4). So far, the effects of histamine on spinal motoneurons are thought to mainly rely on H_1 and H_2 subtypes (Constanti and Nistri, 1976; Taylor et al., 1982; Saito et al., 1984; Wu et al., 2012). Nevertheless, H_3 histamine receptors (H_3Rs) also play a functional role in the spinal cord (Harasawa, 2000; Hough and Rice, 2011) and may thus modulate ventral spinal neuron activity, as well. We explored this hypothesis using *in vitro* neonatal rat spinal cords, which allow to examine spinal and locomotor functions at both circuit and cellular levels (Brumley et al., 2017). H_3Rs were studied on functionally-identified motoneurons using the selective pharmacological agonist for H_3Rs, α-methylhistamine, at a concentration similar to what previously reported on *in vitro* CNS preparations (Brown et al., 1996; Takei et al., 2012; 2017). Histological tools and selective staining assessed the presence of H_3Rs on large-diameter (>15 μm) cells in the ventral horns, while electrophysiological recordings from lumbar ventral roots (VRs) explored the involvement of H_3Rs in modulating the output of lumbo-sacral networks.

Experiments were performed on 39 isolated spinal cords of postnatal day (P) 0 - 4 rats, as previously reported (Dose et al., 2014; 2016). Procedures were approved by the International School for Advanced Studies (SISSA) ethics committee and are in accordance with the guidelines of the Italian Animal Welfare Act 24/3/2014 n. 26 implementing the European Union directive on animal experimentation (2010/63/EU). All efforts were made to minimize number and suffering of animals used. Cords were isolated from the midthoracic region to the *cauda equina* and placed in a small recording chamber at room temperature to be continuously superfused (5 mL/min) with a Krebs solution, composed as follows (in mM): 113 NaCl, 4.5 KCl, 1 $MgCl_2 7H_2O$, 2 $CaCl_2$, 1 NaH_2PO_4, 25 $NaHCO_3$, and 11 glucose, gassed with 95 % O_2-

5 % CO_2, pH 7.4. Tight-fitting suction electrodes allowed DC-coupled extracellular recordings from lumbar ventral roots (VRs; Dose et al., 2016). Intracellular recordings with sharp electrodes (electrode resistance = 38.82 ± 9.98 MΩ) were obtained from 34 motoneurons total, impaled from both left (l) and right (r) L3 - L5 segments (Dose et al., 2014). In control conditions, cells' overall average resting potential was – 66.63 ± 9.22 mV, with membrane resistance of 32.03 ± 14.38 MΩ and antidromic spike amplitude of 65.50 ± 9.32 mV. Selective histaminergic agents were added to the superperfusing medium as needed. Histamine dihydrochloride (Murakoshi et al., 1985) and R-α-methylhistamine dihydrobromide (Dai et al., 2006) were purchased from Tocris (Bristol, UK). Thioperamide maleate (Dai et al., 2006), and tetradotoxin (TTX) were bought from Ascent Scientific (Bristol, UK).

Histology and immunohistochemistry were performed on freshly isolated spinal cords fixed in 4% paraformeldehyde (Fluka, Buchs, Switzerland) for 3 days. Samples were soaked overnight in cryoprotecting 30% sucrose (Fluka) water solution and embedded in OCT (Kaltek, Padova, Italy). From L4 level, serial 15 µm thick cross cryosections were cut rostrocaudally with a 2800 Frigocut N cryostat microtome (Reichert-Jung GmbH, Nussloch, Germany) and mounted on polylysine (Sigma-Aldrich, St. Louis, MO, USA) precoated slices. Serial sections were processed alternatively for staining with 0.1% cresyl violet acetate (Sigma) water solution or H_3R selective immunostaining (Suppl. Fig. 1). For immunohistochemical analysis, slices were rinsed in PBS 0.1 M and then sections were incubated in 0.3% H_2O_2 (BDH Laboratory Supplies, Poole, UK) solution in PBS, at room temperature, in order to block endogenous peroxidase activity. After washing, the sections were blocked with 3% normal goat serum (Vector Laboratories, Burlingame, CA, USA) and 0.25 % Triton X-100 (Bio-Optica, Milano, Italy) PBS solution for 2 hours and then incubated overnight with polyclonal antibody NLS476, RRID:AB_2264153 (Cricco et al., 2008; Novus Biologicals, Littleton, CO, USA) at a dilution of 1:200, in a humidified chamber, at room temperature. After washing, sections were incubated 2 hours in biotinylated secondary antibody (Vector Laboratories) at a dilution of 1:200, and then rinsed. The slices were incubated in ABC solution (Vector Laboratories) for 1 hour at room temperature. Bound antibodies were detected using DAB solution (Vector Laboratories). After immunohistochemical labelling, slices were then counterstained with haematoxylin to visualize cell nuclei (Suppl. Fig. 2). Control experiments included omission of the primary antibody. All slices were subjected to ethanol/xylene (Carlo Erba, Milan, Italy) cleansing and then examined via photomicroscopy (Zeiss, Gotingen, Germany). Large cells in lamina IX (putatively motoneurons) were morphologically identified as cells with a diameter greater than 15 µm. The number of H_3R-positive large cells in ventral horns was calculated

based on immunostained sections (n = 84), whereas total number of large cells in ventral horns was calculated based on cresyl violet stained sections (n = 84), in a 350 x 400 µm area, using ImageJ software.

Mean values from each spinal cord (P2-P4; n = 5) were then obtained. Since no differences were found between right and left spinal cord sides, mean values of those data were calculated and normality was assessed by means of Shapiro-Wilk test ($p > 0.05$).

As for statistical analysis, all data are indicated as a mean ± SD, while *n* indicates the number of cells or spinal cords analyzed. Data were distinguished between parametric and non-parametric, using a normality test. Then, all parametric values were analysed with either Student's t-test (paired or unpaired) to compare two groups of data, or with ANOVA when groups were more than two. Non-parametric data was analysed with a Mann-Whitney test for two groups or, for multiple comparisons, with one-way ANOVA on ranks (Kruskal-Wallis) first, followed by a post hoc test (Dunnett's Method, Tukey Test). Results were considered significant when $p < 0.05$.

To verify the presence of functional H_3Rs in the spinal cord, we added the endogenous agonist, histamine, to the superperfusing medium. An immediate depolarization (about 15 mV) was observed, associated with the superimposed firing activity (Figure 1A, left) previously reported (Coslovich et al., 2018). After an extensive washout from histamine (at least 15 min), the application of α-methylhistamine (20 µM) induced an appreciable depolarization (about 4 mV), accompanied by action potentials (Figure 1A, right). Serial applications of histamine and α-methylhistamine revealed that depolarizations induced by the first were significantly higher than the ones elicited by α-methylhistamine on the same cells (paired t-test, $p = 0.03$, n = 3).

A depolarization of 4.40 ± 1.34 mV was recorded in 10 out of 34 recorded motoneurons, after 154.03 ± 91.31 s from α-methylhistamine application. As data were collected from animals very close in age (P1-P3), the observed sensitivity to the agent in the 29% of recorded motoneurons did not appear to depend upon developmental stage, but rather upon different cell properties. In control conditions, the two groups of cells (sensitive and non-sensitive) showed similar membrane resistance (33.34 ± 16.91 mΩ, sensitive vs. 30.70 ± 13.85 mΩ non-sensitive), but different resting membrane potentials (-59.73 ± 7.53, sensitive vs. -68.68 ± 9.38, non-sensitive; t-test; $p = 0.010$).

To verify the presence of functional H_3Rs on the motoneuron membrane, we blocked the action potential-mediated transmission with TTX (0.5-1 µM) before and during α-methylhistamine application (20 µM). In control conditions, TTX suppressed spontaneous tonic activity, abolished action potentials, and hyperpolarized cells. Addition of α-methylhistamine (20 µM;

Figure 1B) significantly depolarized 60% of motoneurons (mean depolarization 1.23 ± 0.79 mV, paired t-test, p = 0.012, n = 6). In the presence of TTX, the extent of depolarization induced by α-methylhistamine was significantly smaller than the one induced by histamine (Figure 1C, paired t-test, p = 0.019, n = 5, 6).

Since only a subgroup of cells showed to possess H_3Rs, we examined their selective contribution to the overall output of motoneuronal pools. Thus, VR activity was extracellularly recorded during bath-application of H_3R agonist, α-methylhistamine. In a sample cord in Fig. 1D, VRs were depolarized by 367 μV, in accordance with the mean depolarization of 415 ± 64 μV obtained from 11 experiments, where the selective activation of H_3Rs did not elicit any alternating activity from VRs.

In the presence of TTX, histamine (n = 8) and α-methylhistamine (n = 6) were always able to depolarize VRs (Figure 1E). This provides evidence that H_3Rs, although functionally expressed only by a third of cells, once activated, play an important role in modulating overall spinal motor output. In additional five preparations, co-application of histamine, TTX and the selective antagonist for the H_3R subtype, thioperamide, statistically reduced the depolarization induced by histamine (Figure 1E; one-way ANOVA followed by all pair-wise multiple comparison procedures with Tukey test; p = 0.005). The observation showed that H_3Rs mediate part of the effects of histamine application.

In summary, data indicate that, although H_3Rs were functionally involved in modulating the 29% of intracellularly-recorded motoneurons, their role is predominant as the overall VR motor output was indeed depolarized by H_3R selective agents.

Because electrophysiological experiments with TTX strongly indicated the presence of H_3Rs on motoneuron membranes, we performed immunohistochemical labelling to visualize the distribution of H_3Rs in the ventral spinal cord. Interestingly, H_3Rs were expressed on the membrane of 35% of the large diameter (>15 μm) cells in lamina IX, as morphologically confirmed using cresyl violet staining (Figure 2, n = 84 cryosections from 5 spinal cords).

Indeed, α-methylhistamine in TTX affected the activity of only a portion of functionally-identified motoneurons, in line with the expression of H_3Rs on the membranes of one third of large cells in the ventral horn, immunohistochemically labelled.

The identification of H_3Rs on motoneurons explains part of the motor behavior following pharmacological manipulation with selective histamine agents (Chiavegatto et al., 1998; Toyota et al., 2002) and opens a new path in exploring therapeutics to alleviate spinal cord pathologies. Histamine is well known to be involved in nociceptive and antinociceptive processing (Wei et al., 2016) and itch sensations (Lee et al., 2016; Luo et al., 2015). In

particular, H_3Rs, acting on sensory afferents and sensory-responsive neurons in the spinal cord (Cannon et al., 2007), attenuate mechanically-induced nociception (Cannon et al., 2003). Moreover, H_3R antagonism has been suggested as a potential mechanism for the treatment of neuropathic pain in preclinical rat models (Cowart et al., 2012; Hsieh et al., 2010). Our findings suggest that H_3R agents also act on motoneurons in the ventral spinal cord to influence motor behavior and can represent promising pharmacological targets in the CNS, because of their characteristic of being constitutively active *in vivo* (Passani and Blandina, 2011). A direct effect of H_3Rs on interneuronal spinal networks for locomotion should be excluded, since no locomotor-like alternating patterns (Taccola a Nistri, 2005) were elicited by α-methylhistamine alone. The inverse agonism of these receptors unveils their ability to modulate neuronal excitability, even in the absence of any endogenous histamine, as shown by innovative molecules that underwent preclinical testing for the treatment of cognitive disorders and feeding behavior (Arrang et al., 2007).

Recently, histamine has been reported to improve long-term recovery in a rat model of spinal injury, by decreasing the lesion area, inhibiting astrogliosis and glial scar formation, and improving locomotor recovery (Zhao et al., 2015). Although many of the effects were reversed by a treatment with a H_1 receptor antagonist, the authors suggested that the overall protective role of histamine may be due to different histamine receptors in different cell types. Then, the modulation of H_3Rs expressed in a subpopulation of large ventral horn cells could represent a potential neuroprotective strategy to rescue spinal tissue after damage (Kukko-Lukjanov et al., 2006).

Compliance with Ethical Standards

Ethical Approval: Procedures were approved by the International School for Advanced Studies (SISSA) ethics committee and are in accordance with the guidelines of the Italian Animal Welfare Act 24/3/2014 n. 26 implementing the European Union directive on animal experimentation (2010/63/EU). All procedures performed in studies involving animals were in accordance with the ethical standards of the SISSA Laboratory Animal Center and the protocol was approved by the local Animal Committee (Organismo preposto al benessere degli animali, OPBA) of SISSA, Italy.

Conflict of interest: no conflicts of interest to declare

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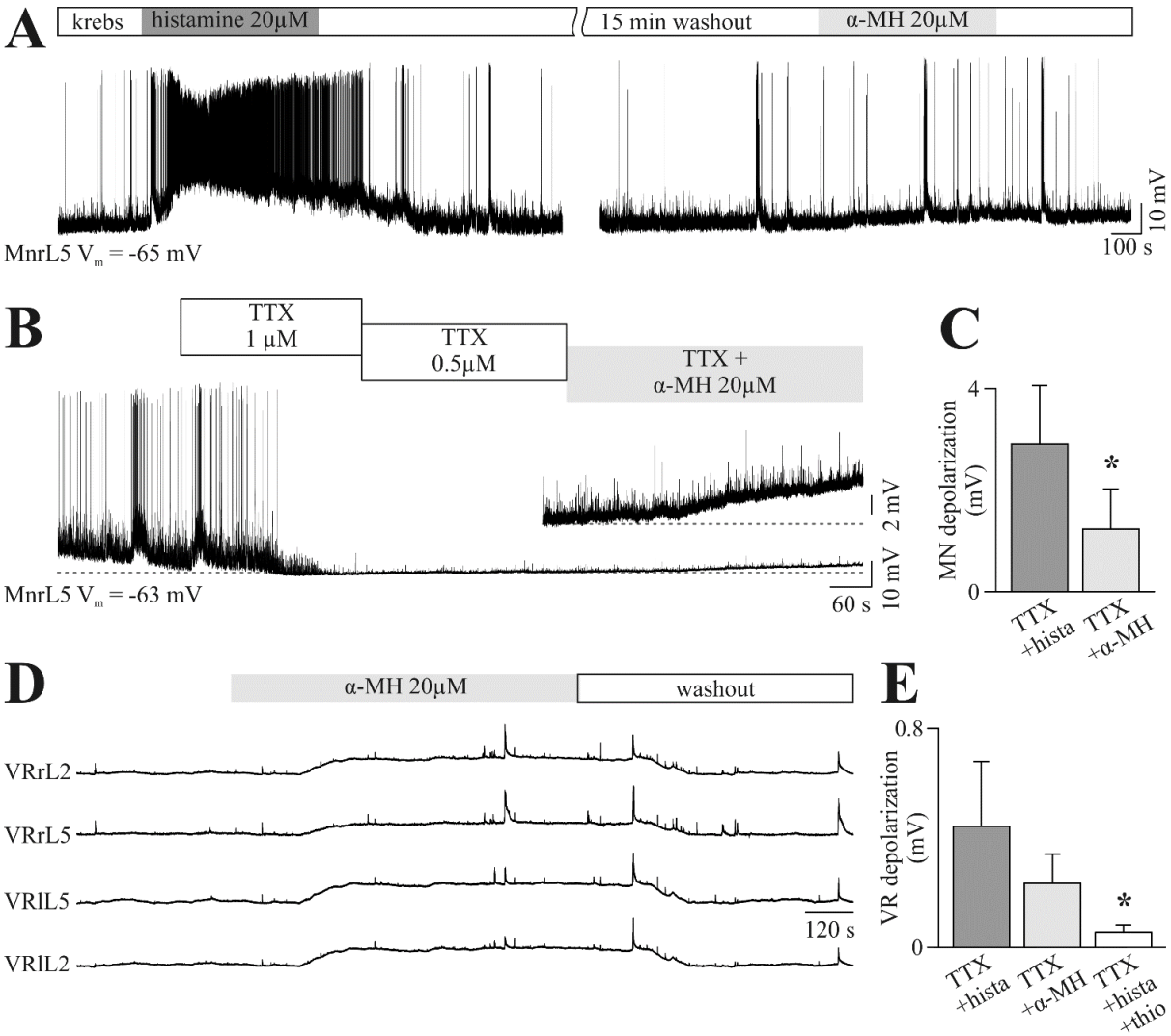


Figure 1. α-methylhistamine directly depolarizes a subset of recorded motoneurons and affects the motor output recorded from VRs.

A.) After 84 s from the application of histamine (upper gray bar; 20 μM), a sustained depolarization (15.32 mV) with superimposed intense firing (3.97 Hz) is recorded from a rL5 single motoneuron (resting potential = -65 mV). After 15 min of washout, the baseline returns to control level and only sporadic action potentials can be derived (trace break = 2 min). Application of α-methylhistamine (α-MH) slightly depolarizes the same cell (4.15 mV) with the occurrence of faster spiking activity. Based on the extent of depolarization induced by 20 μM α-methylhistamine (α-MH), single motoneurons are *a posteriori* divided in sensitive and non-sensitive. B.) A different rL5 motoneuron (- 63 mV initial resting potential, V_m) is hyperpolarized (- 4.94 mV) by TTX (1 and then 0.5 μM) with the suppression of both spontaneous firing and tonic activity, as well. α-methylhistamine (α-MH, 20 μM) slightly depolarizes the motoneuron (2.56 mV) as indicated at a higher magnification in the insert

above. C.) Analysis of pooled data from different experiments during TTX perfusion indicates that the mean depolarization elicited by histamine (20 µM; dark gray bar) is significantly higher than the one induced by α-methylhistamine (20 µM; light gray bar; *, t test; p = 0.019; n = 5, 6). D.) Application of α-methylhistamine (20 µM, 15 min application) induces comparable stable depolarizations with sporadic synchronous events among all VRs (VRrL2 = 349 µV; VRrL5 = 452 µV; VRlL5 = 321 µV; VRlL2 = 347 µV). After 5 min of the following washout, traces return to baseline and discharges are progressively attenuated, until mostly suppressed. E.) Histamine (20 µM) and α-methylhistamine (α-MH; 20 µM) depolarize VRs in the presence of TTX, while the selective H_3R antagonist thioperamide (20 µM) abolishes the depolarization induced by histamine (*, one-way ANOVA followed by all pair-wise multiple comparison procedures with Tukey test; p = 0.005; n = 8, 6, 5). Note that A and B traces come from different motoneurons.

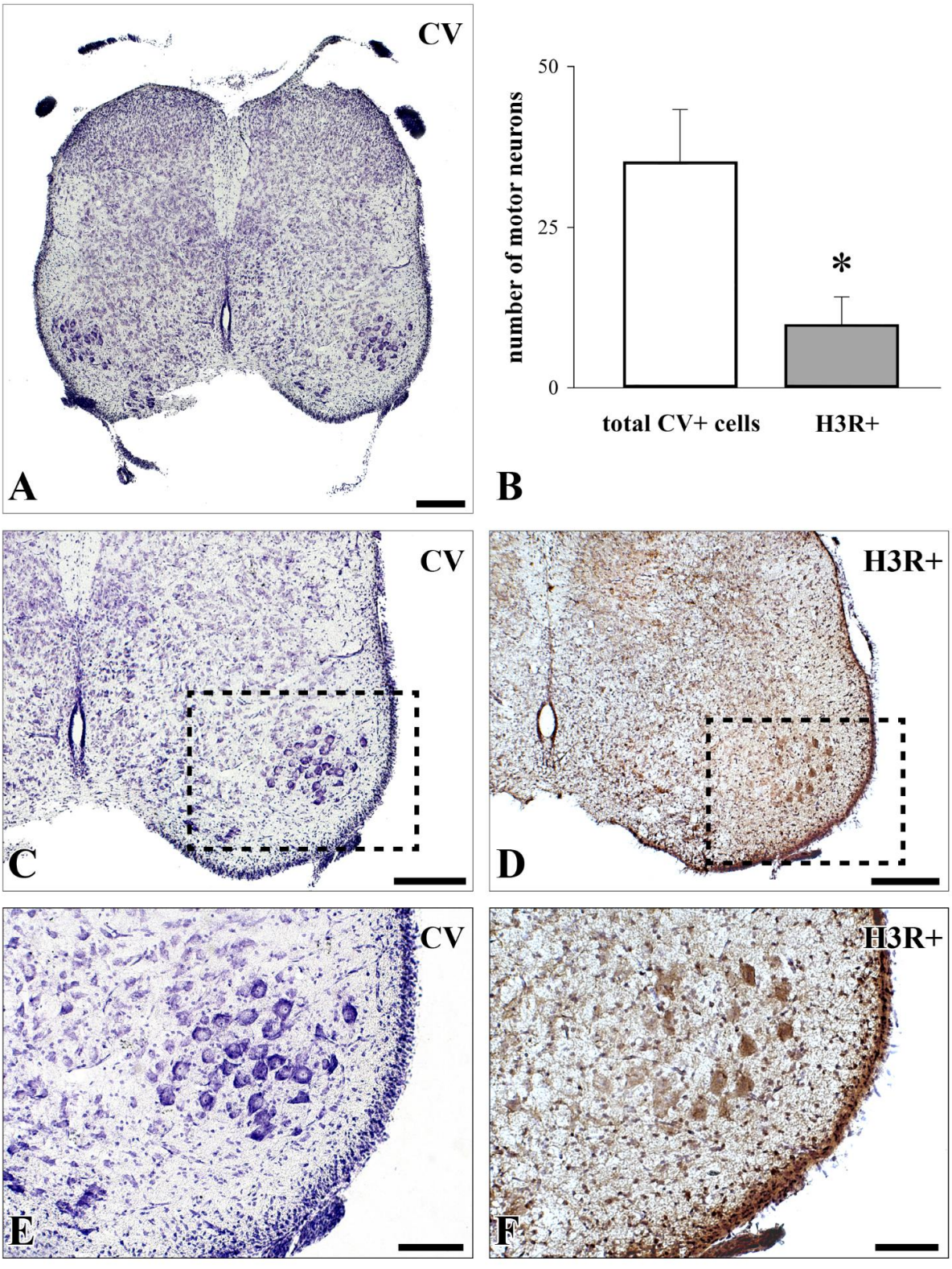
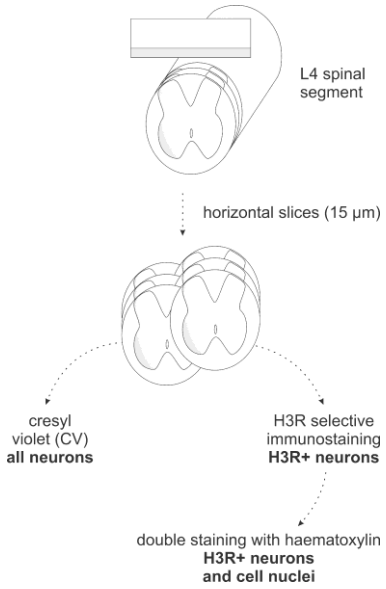

CV
A
50
25
0
number of motor neurons
*
total CV+ cells
H3R+
B
CV
C
H3R+
D
CV
E
H3R+
F

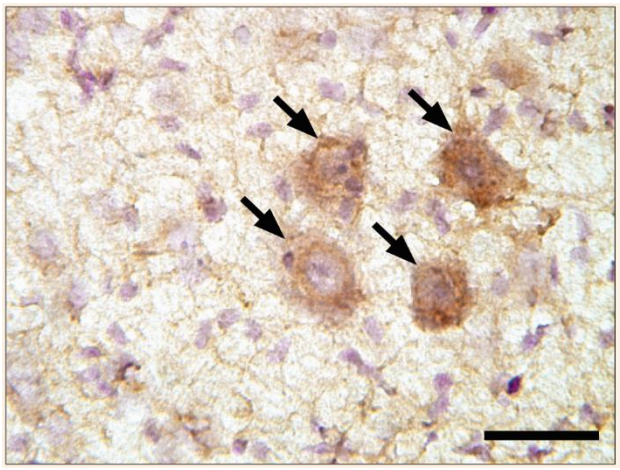
Figure 2. Immunostaining confirms that a subgroup of large cells in lamina IX contains H_3Rs. A.) Cresyl violet staining of 15 µm thick cross cryosection of L4 spinal cord. Dotted rectangle delimiting lamina IX, in which large diameter (>15 µm) ventral horn cells are morphologically identified, is shown in C.) and E.) at a higher magnification. Scale bars: 250 µm A.), 200 µm C.) and 100 µm E.), respectively.

D.) Immunohistochemical detection by H_3R marker on a serial slice from the same cord reveals diffuse labelling concentrated around a set of large ventral horn cells. Dotted rectangle delimiting lamina IX, is shown in F.) at a higher magnification. Scale bars: 200 µm D.) and 100 µm. F.)

B.) Quantification of mean number of H_3R-positive cells among the total number of large ventral horn cells in lamina IX (motor neurons). Large cells in lamina IX were morphologically identified as cells with a diameter greater than 15 µm. Number of H_3R-positive cells is significantly different from the total number of cells, morphologically identified in cresyl violet stained (CV+) sections (*; Student's t-test, $p < 0.001$, $n = 84$ sections from five different cords).



Supplementary Figure 1. The cartoon schematizes the histological processing. Spinal cords, fixed in PFA, were cryosected rostrocaudally from L4 level. Serial 15 µm thick sections were processed alternatively for staining with cresyl violet or H_3R selective immunostaining. After immunohistochemical labelling, slices were then counterstained with haematoxylin to visualize cell nuclei.



Supplementary Figure 2. Double staining by H_3R antibody and haematoxylin shows the expression of H_3R on four large lamina IX cells (black arrows). Scale bar: 50 µm.

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